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Award Number: DAMD17-00-1-0554

TITLE: The Functional Role(s) of Neurofibromin During Neural Crest Cell Development

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REPORT DATE: August 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Final (1 Aug 00 - 31 Jul 02)

4. TITLE AND SUBTITLE
The Functional Role(s) of Neurofibromin During Neural Crest Cell Development

5. FUNDING NUMBERS
DAMD17-00-1-0554

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This grant proposal set out to determine if neurofibromin plays an important role during early neural crest cell development. The objectives of this study were: A) set-up and maintain a viable colony of Nf+/- mice to generate Nf +/+, Nf +/- and Nf-/- embryos. B) Set up a breeding protocol that provides accurately timed pregnant E9 embryos. C. Devise assays to reproducibly assay neural crest cell proliferation and cell death. D. Fix, freeze and collect enough E9 Nf +/+, Nf +/- and Nf-/- embryos with which to generate the relevant data. E. Serially section the embryos in D and perform the proliferation and cell death assays in C to determine whether or not neurofibromin modulates neural crest cell proliferation and cell death in these embryos. To date objectives A-D have been accomplished. Objective E is ongoing and should be completed over the next 6-9months.

14. SUBJECT TERMS
neurofibromatosis, cell development, E9 embryos

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction.

The main purpose of this study is to elucidate on the possible involvement of the neurofibromin protein during neural crest cell development. To achieve this central aim, I will be comparing parameters including NCC proliferation and survival in wild-type (Nf+/+), neurofibromin heterozygote (Nf+/-) and homozygote 'knockout' (Nf-/-) embryos, to see whether or not they differ between these different types of embryo.

Body

The first priority of this study is to establish a healthy and viable colony of Nf+/- mice with which to generate Nf-/- embryos. For a number of reasons this took longer than anticipated. Firstly, I tried to generate Nf+/- mice quickly by mating Nf+/- males and females together. However, I found that the initial batch of the Nf+/- females cannibalized their newborn offspring during the first day of birth. Subsequently, I had more success in generating Nf+/- by mating Nf+/- males with wild-type females since the latter behave less 'quirky'and tended not to kill their progeny. Secondly, the breeding ability of the mice has been adversely affected by 1) outbreaks of mite infection in the breeding room and 2) ongoing construction work here at Children's Hospital of Philadelphia. The latter caused major vibrations perturbing the breeding habits of mice colonies in the animal facility, including my own. To help negate these negative effects on the colony, the Children's Hospital of Philadelphia provided me with ammonia-free, ventilated cage racks. This has markedly improved breeding within the colony. The colony also suffered attrition in Nf+/- females whereby females identified as pregnant by virtue of a vaginal plug, were sacrificed to provide E9 embryos and subsequently found not to be pregnant. Another form of attrition occurred when pregnant dams contained wild-type and Nf+/- but no Nf-/- embryos presumably because they died earlier during development, prior to E9, due to neurofibromin deficiency. However, despite these problems I have performed enough experiments to develop methodologies to assay neural crest cell mitosis and survival in situ (see below), and I have enough Nf-/- E9 embryos (fixed and frozen) to complete this study. I have also developed a more regimented method of obtaining timed E9 embyos. Typically mice are bred overnight and if a vaginal plug is found the next morning, the embryos are timed as E 0.5, assuming that copulation occurred at midnight. In reality, I found that this produces embryos varying in age by as much as 16 hours, i.e the mice copulated anywhere between being placed together in the afternoon or as late as the following morning. Therefore, to obtain accurate and reproducible E9 embryos, the mice were put together at 10am and checked for plugs at 12pm. If no plugs were present at 12pm, the animals were kept together for another 2 hours and if no plug is found at 4pm, the animals were separated and the regime repeated

on the following day. This procedure proved necessary to obtain accurate timed pregnant embryos.

Recent studies have shown that Nf is expressed in a number of cell-types, including NCC, during embryonic development (Daston and Ratner, 1992; Huynh et al., 1994; Stocker et al., 1995). It follows therefore, that the expression of decreased levels of Nf-1 in NCC during development, may have profound effects on the development of NCC and hence NCC-derived tissues. Given my considerable experience in studying NCC development in vitro, I therefore performed pilot experiments in studying the proliferation rates of mouse NCC derived from wild-type, Nf+/-, and Nf-/- E9 embryos in culture. From these experiments, I was greatly concerned by the time involved in isolating, cleaning and harvesting the initial starting tissue (caudal segment of the embryo). In a normal experiment to set-up NCC cultures from a litter of embryos, the embryos are pooled and the dissection time for harvesting caudal segments of the embryos from the first to the last embryo of a litter is typically less than 20min. However, when dissecting and processing tissue embryos individually, and also obtaining tissue for genotyping, the time between obtaining the first to the last embryo in the litter extended to approximately 120min. This variability in time interval most likely explains the large discrepancies in proliferation rate in NCC derived from embryos in same litter. For example, in one experiment the proliferation rate of NCC, as measured by the uptake of bromodeoxyuridine (BrdU), was found to be15.3, 24.7, 25.3, 31.9, 33.6, 41.7, 41.9, 46.9 in 8 of Nf+/- mice derived from the same litter. NCC derived from a single Nf-/- embryo in this litter had a BrDu uptake rate of 31.0. Compared to previous studies on NCC proliferation (Bannerman and Pleasure, 1993, Bannerman et al, 2000, the above wide variability in values is experimentally unacceptable. To circumvent this technical problem and moreover, to analyze the development of NCC in the actual animal, I have developed strategies to analyze NCC proliferation and cell death in situ. In this paradigm, the tissue from individual embryos is immediately fixed upon harvesting and therefore the integrity of the first embryo is preserved rather than being stored in tissue culture medium for up to 120min as part of the initial dissection phase in the in vitro paradigm. The fixed embryos are then processed for cryostat sectioning. Individual embryos are serially sectioned onto a single slide and processed for immunohistochemistry using a mouse monoclonal antibody to AP2, which labels the nucleus of NCC, and rabbit polyclonal antibodies recognizing the phosphorylated form of histone 3 (a nuclear marker for cells undergoing mitosis, see Figure 1. The use of the latter antibody over the more typical use of BrdU uptake, circumvents the need to perform a series of experiments to determine how long a pregnant mouse must be injected with concentrated Brdu in order to label dividing NCC in the embryos via placental transfer. This in situ analysis has been extended to identify NCC undergoing apoptosis in wild-type, Nf+/- and Nf-/-. These experiments will determine whether or not neurofibromin modulates NCC survival. In

this analysis, the TUNEL procedure will be used in conjunction with Ap2 immunohistochemistry to identify NCC undergoing apoptosis. Below I have formally written up the above in situ methodology as it will appear in a peer-reviewed Journal.

Methodology.

Frozen Sections

Timed pregnant E9 embryos were dissected free of their uterus, placenta, chorion and amnion and their heads removed and frozen for genotyping. The remainder of each embryo was fixed in 2% paraformaldehyde for 30min, washed in PBS and cryoprotected overnight in 30% sucrose (in PBS). The embryos were then infiltrated in 2:1 v/v solution of 30% sucrose to OCT cryostat mounting medium (Miles Inc, Kankakee, IL). Prior to sectioning, the embryos were mounted longitudinally on the cryostat chuck into OCT embedding medium at –20°C. The embryos were then cut into 10 μm sections, mounted onto poly-L-lysine coated slides, and stored in slide boxes at -80°C.

Immunofluorescence Studies

I.Proliferation assay--Sections were fixed to slides with 0.5% paraformaldehyde for 1min then pretreated with blocking solution comprised of Minimal Essential Medium (MEM) containing 15mM HEPES buffer, 10% fetal bovine serum and 0.05% sodium azide for 10min. Tissue was then incubated overnight with 3B5 (monoclonal antibody, cultured supernatant used neat) which recognizes AP-2 (Developmental hybridoma Bank) and rabbit anti-phospho-histone3 (mitosis marker, Upstate Biotechnolgy, Lake Placid, NY, 5μg/ml). The tissue was then incubated sequentially with rhodamine conjugated donkey anti-mouse immunoglobulins and fluorescein donkey anti-rabbit immunoglobulins (species specific, Jackson Immunoresearch Labs Inc, West grove, PA, 1:100) for 30min, then post-fixed with cold methanol (–20°C). Cell nuclei were labelled with Hoechst H33258 dye (5μg/ml in PBS) for 5min. Sections were washed between steps PBS and mounted in in vectorshield (Vector Labs, Burlingame, CA).

II.Survival/TUNEL assay—Sections were labelled with 3B5 as described above and then underwent the TUNEL procedure of Gavrieli et al, (1992) with Steptavidin-fluoresein substituted for streptavidin-peroxidase.

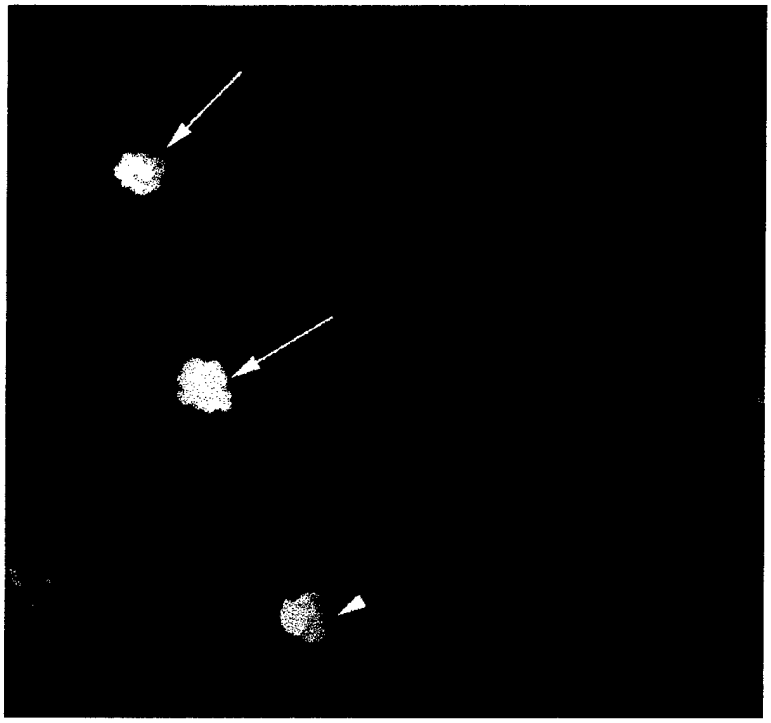

Figure 1 Detection of neural crest cells undergoing mitosis in E9 wild-type embryo. Double label fluorescence analysis shows the nuclei of discrete migrating AP-2^{+} neural crest cells (red), two of which express phosphorylated histone 3 (green, arrows, note these nuclei have a yellow appearance due to overlay of red and green) a marker of cells undergoing mitosis. The arrowhead in this rostral longitudinal 10 micron section points to a cell that is not a neural crest cell, but is undergoing mitosis.

Key Research Accomplishments:

The objectives of this study were: A) set-up and maintain a viable colony of Nf+/- mice to generate Nf +/+, Nf +/- and Nf-/- embryos. B) set up a breeding protocol that provides accurately timed pregnant E9 embryos. C) devise assays to reproducibly assay neural crest cell proliferation and cell death. D) fix,, freeze and collect enough E9 Nf +/+, Nf +/- and Nf-/- embryos with which to generate the relevant data. E) Serially section the embryos in D and perform the proliferation and cell death assays in C to determine whether or not neurofibromin modulates neural crest cell proliferation in these embryos. To date objectives A-D have been accomplished. Objective E is ongoing (see figure 1) and should be completed over the next 6-9months.

Reportable Outcomes:.

The development of the in situ paradigms is novel and will be publishable in a manuscript along with data obtained from it in the future.

Conclusions:

The ability of an established tissue culture system to study whether Neurofibromin modulates the proliferation of NCC has been evaluated and found to give erratically variable data. Alternative in situ paradigm has been developed and has been substituted for the tissue culture system, to provide more reproducible data to study the specific aim of this project. Enough wild-type, Nf and moreover Nf-/- E9 embyos have been collected to complete this project. At the completion of this study I will have determined whether or not neurofibromin plays a biological role(s) in the development of NCC

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Appendices:None